Saponarin activates AMPK in a calcium-dependent manner and suppresses gluconeogenesis and increases glucose uptake via phosphorylation of CRTC2 and HDAC5

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This study investigated the molecular mechanism of saponarin, a flavone glucoside, in the regulation of insulin sensitivity. Saponarin suppressed the rate of gluconeogenesis and increased cellular glucose uptake in HepG2 and TE671 cells by regulating AMPK. Using an in vitro kinase assay, we showed that saponarin did not directly interact with the AMPK protein. Instead, saponarin increased intracellular calcium levels and induced AMPK phosphorylation, which was diminished by co-stimulation with STO-609, an inhibitor of CAMKKb. Transcription of hepatic gluconeogenesis genes was upregulated by nuclear translocation of CRTC2 and HDAC5, coactivators of CREB and FoxO1 transcription factors, respectively. This nuclear translocation was inhibited by increased phosphorylation of CRTC2 and HDAC5 by saponarin-induced AMPK in HepG2 cells and suppression of CREB and FoxO1 transactivation activities in cells stimulated by saponarin. The results from a chromatin immunoprecipitation assay confirmed the reduced binding of CRTC2 on the PEPCK and G6Pase promoters. In TE671 cells, AMPK phosphorylated HDAC5, which suppressed nuclear penetration and upregulated GLUT4 transcription, leading to enhanced glucose uptake. Collectively, these results suggest that saponarin activates AMPK in a calcium-dependent manner, thus regulating gluconeogenesis and glucose uptake.

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Type II diabetes mellitus (T2DM) is a chronic metabolic disease caused by insulin resistance, and its worldwide prevalence has dramatically increased over the past few decades.1 Hyperglycemia and hyperinsulinemia in T2DM is primarily caused by uncontrolled hepatic gluconeogenesis and reduced glucose uptake in skeletal muscles.2 Thus, appropriate control of hepatic gluconeogenesis and cellular glucose uptake are critical in the treatment and prevention of T2DM.

Biguanides, thiazolidinediones, and DPP-4 inhibitors have been widely prescribed for the treatment of T2DM.3 Biguanides are known to activate AMP-activated protein kinase (AMPK) and show improvement in the clinical symptoms of T2DM. As previously shown, AMPK is activated by a high cellular AMP-to-ATP ratio and functions as a master regulator of cellular energy homeostasis by regulating various target proteins via phosphorylation.4 In glucose metabolism, AMPK suppresses hepatic gluconeogenesis and increases glucose uptake in both skeletal muscle and adipose tissue, thus contributing to improved blood glucose homeostasis.5 AMPK suppresses hepatic glucose production through the phosphorylation of two major proteins,6,7 cAMP responsive element-binding protein (CREB) regulated transcription coactivator-2 (CRTC2) and histone deacetylase-5 (HDAC5). AMPK suppresses hepatic gluconeogenesis through inhibitory phosphorylation of CRTC2 and HDAC5. These phosphorylation events induce the sequestration of CRTC2 and HDAC5 in the cytoplasm. CRTC2 and HDAC5 are coactivators of CREB and forkhead box O1 (FoxO1), two major transcription factors that induce the transcription of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase.
(G6Pase), major genes in hepatic gluconeogenesis. Thus, AMPK-dependent phosphorylation of CRTC2 and HDAC5 leads to the suppression of gluconeogenesis. Alternatively, the uptake of cellular glucose is facilitated by glucose transporter-4 (GLUT4), the exocytosis to the plasma membrane and gene expression of which are regulated by the action of insulin. GLUT4 gene expression is regulated by the phosphorylation of HDAC5, which acts as its transcriptional repressor. Transcriptional regulation of the GLUT4 promoter includes the myocyte enhancer factor-2 (MEF2)-binding domain, which is inhibited by HDAC5. AMPK-dependent phosphorylation of HDAC5 prevents its nuclear translocation, which in turn leads to the upregulation of GLUT4 gene expression.

Synthetic prescribed drugs are efficient therapeutics for T2DM; however, their side effects can often be serious (e.g., biguanides cause lactic acidosis). Thus, using natural compounds with moderate activity could be a helpful alternative approach for the prevention of T2DM. The volume of publications on this subject has suggested that flavonoids ameliorate the symptoms of T2DM. For example, the intake of flavan-3-ols rich cocoa for 18 weeks improves the homeostatic model assessment index of insulin resistance, which can ameliorate diabetes in human trials.

In this study, we investigate the effect of saponarin (apigenin-6-C-glucosyl-7-O-glucoside) (Fig. 1A), a flavone glucoside. Saponarin is found in diverse plants, including Tinospora cordifolia, which is used in an anti-diabetic drug, Aloe barbadensis, and barley leaves. Recently, we found that the administration of barley sprout extract for 12 weeks lowered fasting glucose levels in the plasma and improved insulin sensitivity in mice fed a high-fat diet. Barley sprout contained saponarin as a major flavonoid (1.1%, w/w), which could improve glucose metabolism and induce insulin sensitivity. Thus, we examined the mechanism of saponarin on gluconeogenesis and glucose uptake using cultured hepatocytes and myocytes.

**Saponarin improves gluconeogenesis and glucose uptake:** Plasma glucose homeostasis is regulated by two major mechanisms: glucose production in hepatocytes and glucose uptake in myocytes. The effects of saponarin on gluconeogenesis and glucose uptake were investigated in HepG2 and TE671 cells, cultured human hepatocytes and myocytes, respectively (Fig. 1).

Glucose production in HepG2 cells stimulated with saponarin in glucose-free media was significantly decreased compared to that in the controls. Glucose production was reduced by 56% in the presence of 100 μM of saponarin (Fig. 1B), and this reduction was greater than the effect of metformin (1 mM). In myocytes, saponarin treatment for 24 h induced glucose uptake regardless of the presence of insulin. For example, glucose uptake in TE671 cells increased by +72% at 100 μM of saponarin and glucose uptake was further increased to +96% in the presence of insulin (Fig. 1C). These results suggest that saponarin has a synergistic effect with insulin on glucose uptake.

**Saponarin regulates AMPK through induction of intracellular calcium levels:** Hepatic gluconeogenesis and muscular glucose uptake are both regulated by AMPK activity. Thus, we investigated the effects of saponarin on AMPK activation. Through immunoblotting

![Figure 1](image-url)
analysis, we observed that saponarin induced the phosphorylation of AMPK at Thr 172, which is known to lead to AMPK activation (Fig. 1D). Next, the mechanism of AMPK activation by saponarin was investigated. First, using a cell-free kinase assay, we found that saponarin did not bind directly to AMPK to activate it, whereas A-769662, a synthetic agonist of AMPK, directly activated AMPK allosterically ($EC_{50} = 576$ nM, Fig. S1). Thus, saponarin is not an allosteric regulator of AMPK. Second, AMPK activity can be activated by intracellular calcium levels through the activation of an upstream kinase, CAMKK-$\beta$. Our results assessed by a cellular calcium assay showed that saponarin increased the calcium concentration in both HepG2 and TE671 cells (Fig. 2A) and that the induction of AMPK phosphorylation by saponarin disappeared in the presence of STO-609, an inhibitor of CAMKK-$\beta$ (Fig. 1D). These results suggest that saponarin activates AMPK phosphorylation in a calcium-dependent manner.

Saponarin suppresses CREB and FoxO1 activity in cultured hepatocytes: AMPK suppresses hepatic gluconeogenesis through inhibitory phosphorylation of CRTC2 and HDAC5. These phosphorylation events induce the sequestration of CRTC2 and HDAC5 in the cytoplasm. CRTC2 and HDAC5 are coactivators of CREB and FoxO1, two major transcription factors that induce the transcription of PEPCK and G6Pase. Thus, AMPK-dependent phosphorylation of CRTC2 and HDAC5 leads to the suppression of gluconeogenesis. In HepG2 cells stimulated with saponarin (100 $\mu$M) for 4 h, we observed significantly increased phosphorylation of both CRTC2 and HDAC5 (229% and 187%, respectively) compared to the controls and thus reduced nuclear CRTC2 and HDAC5 (~51% and ~50%, respectively) via immunoblotting analysis (Fig. 2B). The transactivation of CREB and FoxO1 assessed by luciferase assay were suppressed in cells treated with saponarin (~62% and ~14% reduction, respectively, vs. controls, $P < 0.05$, Fig. 3A). The effect of saponarin was greater on CREB activity. Finally, via a ChIP assay, we observed that saponarin stimulation reduced the binding of CRTC2 to both PEPCK and G6Pase promoters (~28% and ~34% reduction, respectively, vs. controls, Fig. 3B); thus, mRNA expression of PEPCK and G6Pase was downregulated by saponarin (Fig. 3C). These results suggest that saponarin suppresses hepatic gluconeogenesis primarily through the activation of the AMPK–CREB signaling axis, which downregulates PEPCK and G6Pase gene expression.

Saponarin phosphorylates HDAC5 and induces GLUT4 expression in cultured myocytes: GLUT4 expression is controlled by two transcription factors, GEF and MEF2A, both of which are stimulated by AMPK. Specifically, hyperacetylation of the MEF2A promoter is facilitated by the AMPK-dependent phosphorylation of HDAC5. Saponarin-stimulated HDAC5 phosphorylation (+63% in the presence of 100 $\mu$M of saponarin, Fig. 4A), thus reducing nuclear HDAC5 in TE671 cells. Accordingly, the mRNA expression of

![Figure 2](image-url)

Figure 2. Saponarin induces intracellular calcium concentration and phosphorylation of CRTC2 and HDAC5. (A) Saponarin stimulation immediately increases cellular calcium levels. (B) Phosphorylation and nuclear levels of CRTC2 and HDAC5 quantified by immunoblotting analysis. The different letters denote significant differences among the groups assessed by one-way ANOVA followed by the Tukey test ($P < 0.05$). Data are represented as the mean ± SEM. Con, vehicle control; A769, A769662 at 100 $\mu$M; S50, saponarin at 50 $\mu$M; S100, saponarin at 100 $\mu$M.
GLUT4 was upregulated in cells stimulated with saponarin (Fig. 4B). These results suggest that saponarin induces glucose uptake by activation of GLUT4 gene expression.

Saponarin is a flavone glucoside that is found in some plant leaves, including barley sprout, Aloe vera, Cucumis sativus, and mosses. Saponarin has been shown to be involved in several biological activities, including hypoglycemic effects and the improvement of insulin sensitivity. However, its molecular mechanisms of action have been elusive. In this study, we suggest that saponarin strongly activated AMPK phosphorylation via CAMKKβ, which was confirmed by STO-609, a CAMKKβ inhibitor. Saponarin did not interact directly with the AMPK protein, thus it is not an allosteric regulator. Activation of AMPK led to the phosphorylation of transcription modulators CRTC2 and HDAC5, which prevented their transport into the nucleus. This prevented CREB- and FoxO1-dependent transcription of PEPCK and G6Pase in hepatocytes and suppression of GLUT4 by HDAC5 in myocytes. The results of this study revealed that saponarin induced phosphorylation of CRTC2 and HDAC5, which decreased the expression of gluconeogenesis genes and increased the expression of GLUT4 in myocytes.

Activation of AMPK enhances insulin sensitivity, stimulating glucose uptake in muscle and adipose tissues and inhibiting glucose production in the liver; thus, this is an important strategy in the control of type II diabetes. The biguanide metformin is a widely prescribed insulin sensitizer that activates AMPK in multiple tissues. Metformin lowers plasma glucose and lipid levels through the reduction of hepatic gluconeogenesis and induction of glucose uptake in muscle tissue. Our results strongly indicated that saponarin activates AMPK via the calcium–CAMKKβ pathway and phosphorylates AMPK to stimulate its downstream targets.

PEPCK and G6Pase are the key genes in hepatic gluconeogenesis, and their expression is positively correlated with the development of type II diabetes. CREB and FoxO1 regulate these two genes. AMPK phosphorylates CRTC2 and HDAC5, transcriptional coactivators of CREB and FoxO1, respectively, in gluconeogenesis. Phosphorylated CRTC2 and HDAC5 are blocked from nuclear translocation and thus cannot participate in CREB- and FoxO1-dependent transcription of PEPCK and G6Pase. Thus, activation of AMPK reduces hepatic gluconeogenesis and glucose output to circulation. We confirmed that saponarin suppressed gluconeogenesis in cultured HepG2 cells. (A) Transactivation of CREB and FoxO1 measured by the luciferase assay. (B) Binding of CRTC2 to the promoter of PEPCK and G6Pase genes assessed by the ChIP assay. (C) mRNA expression of the PEPCK and G6Pase genes assessed by qPCR analysis. The mRNA expression levels were compared with those in the control group as a reference. The different letters denote significant differences among the groups performed by one-way ANOVA followed by the Tukey test (P<0.05). Data are represented as the mean ± SEM. Con, vehicle control; A769, A769662 at 100 μM; S50, saponarin at 50 μM; S100, saponarin at 100 μM.
hepatocytes following AMPK phosphorylation of CRTC2 and HDAC5, which decreased its nuclear levels and thus the transactivation of CREB and FoxO1. Saponarin showed greater suppression on CREB activity compared with that of FoxO1.

GLUT4 is a key gene in glucose uptake in skeletal muscle cells. Its gene expression is regulated by two transcription factors, GEF and MEF2A, the activity of which is primarily controlled by AMPK. AMPK phosphorylates GEF directly to activate GLUT4 transcription and phosphorylates HDAC5, which hyperacetylates the MEF2A promoter and induces GLUT4 transcription. The results of this study confirmed that saponarin induces HDAC5 phosphorylation and decreases nuclear HDAC5, thus upregulating GLUT4 mRNA expression. In addition to AMPK-dependent upregulation of GLUT4, CAMKKβ also induces GLUT4 via activating phosphorylation of HDAC5. Because studies have suggested that the activation of both AMPK and CAMKKβ reinforces GLUT4 transcription, the induction of intracellular calcium and activation of CAMKKβ by saponarin may additionally contribute to the induction of GLUT4 gene expression. We found that saponarin significantly increased GLUT4 gene expression levels within 24 h.

Although this study suggests a novel mechanism of saponarin in the regulation of insulin sensitivity in vitro, there are some limitations. Saponarin induced AMPK phosphorylation in a calcium-dependent manner, and not by direct interaction with AMPK proteins. Thus, the molecular target of saponarin in the regulation of intracellular calcium concentrations has not been elucidated in this study. There are many different ways to regulate intracellular calcium concentration, including activation of C_{in} by the G-protein coupled receptor pathways and activation of ligand-gated calcium channels. The mechanism of calcium regulation by saponarin should be investigated further. In addition, the effects of saponarin need to be confirmed in animal and human studies. These remaining questions and experiments should be pursued in future studies.

In conclusion, saponarin has a hypoglycemic effect through the activation of the CAMKKβ–AMPK–CREB signaling axis in hepatocytes. Activation of AMPK and the induction of GLUT4 gene expression contribute to the increase in glucose uptake. These events could collectively improve glucose homeostasis and insulin sensitivity. Future studies in animals and humans will aim to confirm these observations.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.09.057.

References and notes

23. HepG2 and TE671 cells were obtained from the Korean Cell Line Bank (Seoul, K.
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23. HepG2 and TE671 cells were obtained from the Korean Cell Line Bank (Seoul, K. a. g. i. D. H., Logan, UT, USA) supplemented with 10% heat-inactivated fetal
26. Cells were seeded at a density of 30,000 cells/well in black 96-well plates. The
27. Cells were lysed with a 1% protease inhibitor cocktail at 4 °C. The protein
28. HEK293T cells were seeded in 24-well plates at a density of 1 × 10^4 cells/well.
30. HepG2 cells were fixed with 4% formaldehyde after saponarin stimulation. The
cells were collected by centrifugation (2000 × g for 5 min at 4 °C) and washed
twice with cold PBS. An IP buffer containing protease inhibitors was added and the nucleus pellet was collected by centrifugation (12,000 × g for 1 min at 4 °C).
32. Total RNA was extracted from livers and HepG2 cells using RNAiso Plus (Takara, Shiga, Japan). CDNA was synthesized from 2 μg of total RNA using M-MLV Reverse Transcriptase (Mbittech, Seoul, Korea) and oligo(dT) primers.